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Article

Synaptic Glutamate Release by Ventromedial Hypothalamic Neurons Is Part of the Neurocircuitry that Prevents Hypoglycemia

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SUMMARY

The importance of neuropeptides in the hypothalamus has been experimentally established. Due to difficulties in assessing function in vivo, the roles of the fast-acting neurotransmitters glutamate and GABA are largely unknown. Synaptic vesicular transporters (VGLUTs for glutamate and VGAT for GABA) are required for vesicular uptake and, consequently, synaptic release of neurotransmitters. Ventromedial hypothalamic (VMH) neurons are predominantly glutamatergic and express VGLUT2. To evaluate the role of glutamate release from VMH neurons, we generated mice lacking VGLUT2 selectively in SF1 neurons (a major subset of VMH neurons). These mice have hypoglycemia during fasting secondary to impaired fasting-induced increases in the glucose-raising pancreatic hormone glucagon and impaired induction in liver of mRNAs encoding PGC-1α and the gluconeogenic enzymes PEPCK and G6Pase. Similarly, these mice have defective counterregulatory responses to insulin-induced hypoglycemia and 2-deoxyglucose (an antimetabolite). Thus, glutamate release from VMH neurons is an important component of the neurocircuitry that functions to prevent hypoglycemia.

INTRODUCTION

Glutamate and GABA are the major excitatory and inhibitory neurotransmitters in the brain. They exert strong effects on postsynaptic neurons and are widely distributed. With this in mind, it is remarkable how little is known about their roles in transducing specific behaviors and homeostatic responses. This is especially true when one considers the hypothalamus, a site where the majority of focus has been on neuropeptides. Indeed, critically important roles have been established for many hypothalamic neuropeptides and their receptors in regulating food intake, energy expenditure, insulin-glucose homeostasis, the sleep-wake cycle, and neuroendocrine output of the pituitary gland (Cone, 2005; Guillemin, 2005; Saper et al., 2005; Williams et al., 2004). In striking contrast, the roles played by fast-acting neurotransmitters such as glutamate and GABA have been largely unexplored. This is not because they are thought to be unimportant, but instead because of the absence of methodological approaches for testing their function in vivo (van den Pol, 2003). The underlying problem limiting investigations is the widespread distribution of glutamate, GABA, and their receptors. Because essentially all neurons have receptors for these neurotransmitters, site-specific injections of agonists or antagonists invoke responses in all neurons exposed, generating results of unclear physiologic meaning (van den Pol, 2003). Similarly, total gene knockouts aimed at disrupting neurotransmitter release or the ability to respond to neurotransmitters result in complex phenotypes, many of which are incompatible with life (Fremeau et al., 2004a; Moechars et al., 2006; Wallen-Mackenzie et al., 2006; Wojcik et al., 2004, 2006). Thus, there is tremendous need for an approach that allows one to study the roles of glutamate and GABA in selected sites within the brain and, hence, in specific behaviors and homeostatic responses.

Toward these ends, we and others (Wallen-Mackenzie et al., 2006) have created lox-modified alleles of synaptic vesicle transporters. In order for a neuron to release glutamate or GABA, the neurotransmitter must first be packaged, at high concentrations, into synaptic vesicles. This is accomplished by specific transporters, VGLUT1, -2,

and -3 in the case of glutamate (Fremeau et al., 2004b), and VGAT in the case of GABA (Gasnier, 2004). It has previously been demonstrated that knockout of *Slc17a7* (*Vglut1*) (Fremeau et al., 2004a; Wojcik et al., 2004), *Slc17a6* (*Vglut2*) (Moechars et al., 2006; Wallen-Mackenzie et al., 2006), and *Slc32a1* (*Vgat*) (Wojcik et al., 2004) creates neurons that are unable to release their respective neurotransmitter. By creating lox-modified alleles of these synaptic vesicle transporters and by crossing such animals with transgenic mice expressing Cre recombinase in specific groups of neurons, transsynaptic communication by these transmitters can be disrupted in a neuron-specific manner. Such studies make it possible to determine the role of fast-acting neurotransmitters in specific behaviors and homeostatic responses.

The ventromedial hypothalamus (VMH) integrates forebrain neuronal input with ascending information from the brainstem and then sends output to regions involved in control of various behaviors and physiologic responses (Swanson, 1987). Indeed, VMH neurons send projections to a vast number of sites, including numerous locations within the medial hypothalamus, the lateral hypothalamus, the zona incerta, parts of the midline thalamus, the bed nuclei of the stria terminalis, various parts of the amygdala, and the periaqueductal gray (Canteras et al., 1994; Krieger et al., 1979; Saper et al., 1976). The VMH also sends projections, at a lower density, to many other sites, including direct glutamatergic projection to nearby POMC neurons (Sternson et al., 2005). By means of these projections, the VMH is thought to control ingestive, sexual, and defensive behaviors (Canteras, 2002; Choi et al., 2005; King, 2006) and the physiologic responses that accompany these behaviors, as well as counterregulatory responses to hypoglycemia (Borg et al., 1994, 1995, 1997). Neurons in the VMH are largely glutamatergic as evidenced by high-level expression of mRNA encoding the vesicular glutamate transporter VGLUT2 and lower levels of expression of mRNA encoding the vesicular GABA transporter VGAT and the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) (Ovesjo et al., 2001; Ziegler et al., 2002). The importance of glutamate release from VMH neurons versus other neurotransmitters and neuropeptides in mediating the above-mentioned behaviors and physiologic responses, however, is unknown.

Steroidogenic factor 1 (SF1, official gene name *Nr5a1*) is a member of the nuclear hormone receptor family and, in the CNS, is expressed exclusively in the VMH (Dhillon et al., 2006; Stallings et al., 2002). *Sf1* knockout mice have abnormal VMH development and are obese (Majdic et al., 2002). Recently, we generated *Sf1-Cre* transgenic mice that express Cre recombinase selectively in the VMH (Dhillon et al., 2006). These mice are useful for VMH-specific knockout of genes flanked by lox sites. We have used this approach to generate mice lacking leptin receptors (LEPRs) on SF1 neurons (*Sf1-Cre;Lepr*$^{flox/flox}$ mice). These mice develop mild obesity when fed a chow diet and become more obese when fed a high-fat diet (Dhillon et al., 2006). As mentioned above, the relevant mediator released by SF1 neurons is largely unknown. In the present study, we evaluated the role of glutamate by generating mice that lack VGLUT2 selectively in SF1 neurons (*Sf1-Cre;Vglut2*$^{flox/flox}$ mice).

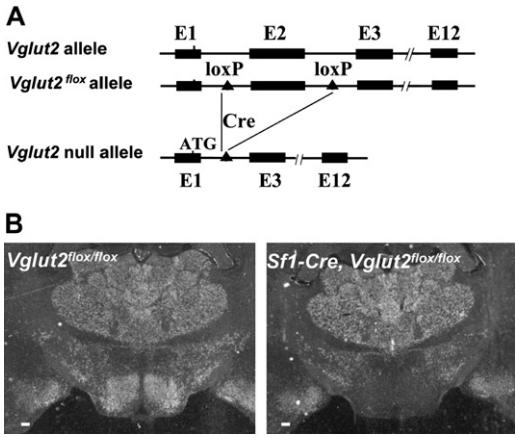


Figure 1. Generation of Mice lacking VGLUT2 in SF1 Neurons
(A) The *Vglut2* gene, the *Vglut2*^{flox} allele, and the Cre-deleted *Vglut2* null allele. E, exon; Cre, Cre recombinase.
(B) In situ hybridization for *Vglut2* mRNA. Scale bar = 100 μM.

RESULTS

Generation of *Vglut2*$^{flox/flox}$ Mice

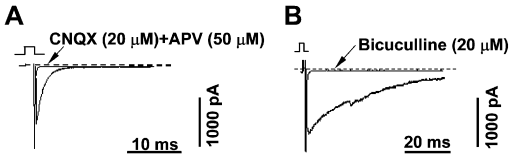
Gene targeting in ES cells was used to create mice with lox sites flanking exon 2 of the *Vglut2* gene (Figure 1A). Cre recombinase-mediated deletion of exon 2 is expected to generate a null *Vglut2* allele, as exon 1 contains only ~5% of VGLUT2's coding sequence. Furthermore, the reading frame is disrupted if exon 1 splices to exon 3. To confirm the null status of this allele, we generated mice homozygous for the deleted allele. The deleted allele was first created by crossing *Vglut2*$^{+/flox}$ mice with transgenic animals expressing Cre in the female germline (*ZP3-Cre* mice; The Jackson Laboratory, stock #003651). Female *ZP3-Cre;Vglut2*$^{+/flox}$ mice were then used to generate offspring that were heterozygous for the deleted allele (*Vglut2*$^{+/-}$ mice). These heterozygotes (*Vglut2*$^{+/-}$) were then bred to generate homozygous, presumably null animals (*Vglut2*$^{-/-}$). Prior studies have demonstrated that mice lacking VGLUT2 are not viable (Moechars et al., 2006; Wallen-Mackenzie et al., 2006). Consistent with this, we were unable to detect any homozygous offspring at age 3 weeks (out of 31 offspring genotyped, 10 were *Vglut2*$^{+/+}$, 21 were *Vglut2*$^{+/-}$, and 0 were *Vglut2*$^{-/-}$). However, we were able to detect offspring homozygous for the deleted allele at embryonic day 19 (out of 10 embryos genotyped, 3 were *Vglut2*$^{+/+}$, 5 were *Vglut2*$^{+/-}$, and 2 were *Vglut2*$^{-/-}$). RT-PCR using mRNA isolated from brain tissue of *Vglut2*$^{+/-}$ mice confirmed that exon 2 was missing and that exon 1 was spliced to exon 3. These results demonstrate that the Cre-mediated deletion of the *Vglut2*^{flox} allele results in a null allele.

Generation of Mice Lacking VGLUT2 in SF1 Neurons

To generate mice lacking VGLUT2 in SF1 neurons, as well as control mice, *Vglut2*$^{flox/flox}$ mice were crossed with *Sf1-Cre;Vglut2*$^{flox/flox}$ mice. To test for neuron-specific loss of *Vglut2* mRNA, in situ hybridization was performed using a riboprobe corresponding to exon 2. As previously observed (Ziegler et al., 2002), *Vglut2* mRNA is abundantly expressed in the VMH of control mice (Figure 1B, left panel). As expected, *Vglut2* mRNA is selectively absent in the VMH of *Sf1-Cre;Vglut2*$^{flox/flox}$ mice (Figure 1B, right panel). In contrast, *Vglut2* mRNA is undisturbed in all other brain regions of *Sf1-Cre;Vglut2*$^{flox/flox}$ mice (data not shown). We were unable to detect VMH expression of *Vglut1* mRNA or *Vglut3* mRNA in control (*Vglut2*$^{flox/flox}$ mice) or VMH-deleted (*Sf1-Cre;Vglut2*$^{flox/flox}$) mice (see Figure S1 in the Supplemental Data available with this article online). Thus, in *Sf1-Cre;Vglut2*$^{flox/flox}$ mice, *Vglut2* mRNA is undetectable in the VMH and there is no compensatory upregulation of *Vglut1* or *Vglut3* mRNA.

Glutamate Release Is Disrupted in SF1 Neurons of *Sf1-Cre;Vglut2*$^{flox/flox}$ Mice

To confirm that deletion of VGLUT2 in SF1 neurons leads to loss of glutamate release, we performed electrophysiologic recordings (whole-cell, voltage-clamp mode) on SF1 neurons, plated at low density, that had formed autosynapses (autaptic cultures) (Hentges et al., 2004). To permit identification of SF1 neurons, *Sf1-Cre* transgenic mice were first crossed with animals bearing a Cre-dependent GFP reporter transgene (*Z/EG*; The Jackson Laboratory) (Novak et al., 2000). Control neurons were derived from *Sf1-Cre;Z/EG* mice, while VGLUT2-deleted neurons were derived from *Sf1-Cre;Z/EG;Vglut2*$^{flox/flox}$ mice. Excitatory (mediated by glutamate) and inhibitory (mediated by GABA) postsynaptic currents (EPSCs and IPSCs) were evoked every 20 s by depolarizing pulses (from a holding potential of −60 mV to +10 mV for 2 milliseconds). With the conditions utilized (see Experimental Procedures), both EPSCs and IPSCs were identified as inward currents. Glutamate-mediated EPSCs versus GABA-mediated IPSCs were differentiated based upon decay time constants (0.8–3.0 ms for EPSCs and 10–15 ms for IPSCs) and effects of glutamate receptor and GABA receptor antagonists. Out of 12 control SF1 neurons, 8 neurons were found to have EPSCs mediated by glutamate (Figure 2A), while 2 neurons were found to have IPSCs mediated by GABA (Figure 2B, results summarized in Figure 2C). Two neurons lacked EPSCs or IPSCs, indicating that they release neither glutamate nor GABA, or that they failed to establish functional autosynapses. These results indicate that most SF1 neurons are glutamatergic while a smaller percentage are GABAergic. Of interest, out of 11 SF1 neurons from *Sf1-Cre;Z/EG;Vglut2*$^{flox/flox}$ mice, 0 neurons were found to have EPSCs, while 2 neurons were found to have IPSCs mediated by GABA (results summarized in Figure 2C). These findings indicate that, as expected, glutamate release is selectively disrupted in SF1 neurons of *Sf1-Cre;Vglut2*$^{flox/flox}$ mice.



C

Genotype	*Sf1-Cre, Z/EG*	*Sf1-Cre, Vglut2*$^{flox/flox}$*, Z/EG*
Total # of neurons recorded	12 neurons	11 neurons
# of neurons with glutamatergic EPSC	8	0
# of neurons with GABAergic IPSC	2	2
# of neurons lacking synaptic current	2	9

Figure 2. Assessment of Neurotransmitter Release in Autaptic Cultures

Control SF1 neurons were prepared from *Sf1-Cre;Z/EG* mice, and VGLUT2-deficient SF1 neurons were prepared from *Sf1-Cre;Z/EG;Vglut2*$^{flox/flox}$ mice.

(A) Excitatory postsynaptic current from a control SF1 neuron recorded in the absence and then presence of glutamate receptor antagonists (CNQX and APV).

(B) Inhibitory postsynaptic current from a control SF1 neuron recorded in the absence and then presence of a GABA receptor antagonist (bicuculline).

(C) Summary of recordings from control (*Sf1-Cre;Z/EG*) and VGLUT2-deficient (*Sf1-Cre;Z/EG;Vglut2*$^{flox/flox}$) SF1 neurons.

Cell Bodies and Projections of SF1 Neurons in *Sf1-Cre;Vglut2*$^{flox/flox}$ Mice

It is conceivable that disruption of glutamatergic transmission could alter the survival and/or projections of SF1 neurons. To address this, we compared the density of cell bodies and projections of control (*Sf1-Cre;Z/EG*) and VGLUT2-deleted (*Sf1-Cre;Z/EG;Vglut2*$^{flox/flox}$) SF1 neurons. The SF1 neurons and their projections were identified by immunohistochemical detection of GFP in brain sections of 10-week-old mice. These studies indicated that the density of SF1 cell bodies in the VMH was unchanged in *Sf1-Cre;Z/EG;Vglut2*$^{flox/flox}$ mice (Figure S2A). To check for projections, we examined the bed nuclei of stria terminalis and the periaqueductal gray area, two sites that are densely innervated by VMH neurons. These analyses indicated that projections were not grossly disturbed in *Sf1-Cre;Z/EG;Vglut2*$^{flox/flox}$ mice (Figure S2B). In total, these studies indicate that glutamatergic transmission is not required for the survival of SF1 neurons or for the development of their projections.

Body Weight in *Sf1-Cre;Vglut2*$^{flox/flox}$ Mice

Sf1-Cre;Vglut2$^{flox/flox}$ mice fed a standard chow diet had normal body weights (data not shown). However, when fed a high-fat, high-sucrose diet, *Sf1-Cre;Vglut2*$^{flox/flox}$ mice developed a modest increase in body weight compared with controls (*Vglut2*$^{flox/flox}$ mice) (Figure 3A). This was associated with a small increase in food intake (Figure 3B) and fat stores (Figure 3C) but not with an obvious effect on energy expenditure (Figure 3D).These results suggest that release of glutamate from SF1 neurons plays

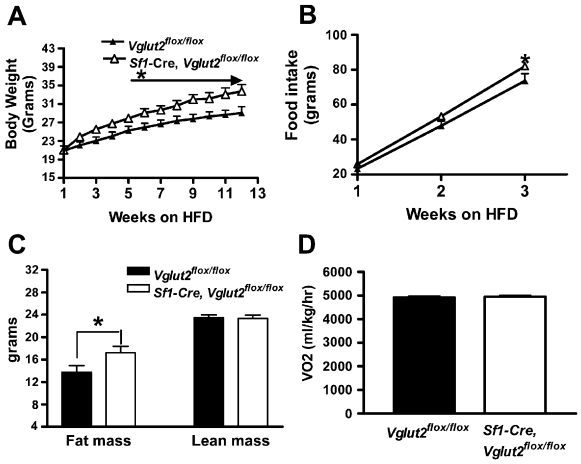


Figure 3. Effect of High-Fat, High-Sucrose Diet on Energy Balance

Male mice (n = 8–10 mice per group) were studied. The high-fat, high-sucrose diet (HFD) was started at the age of 8–10 weeks. In this and all other figures, error bars represent ±SEM. *p < 0.05.

(A) Body weight.

(B) Cumulative food intake.

(C) Body composition.

(D) Energy expenditure as assessed by oxygen consumption.

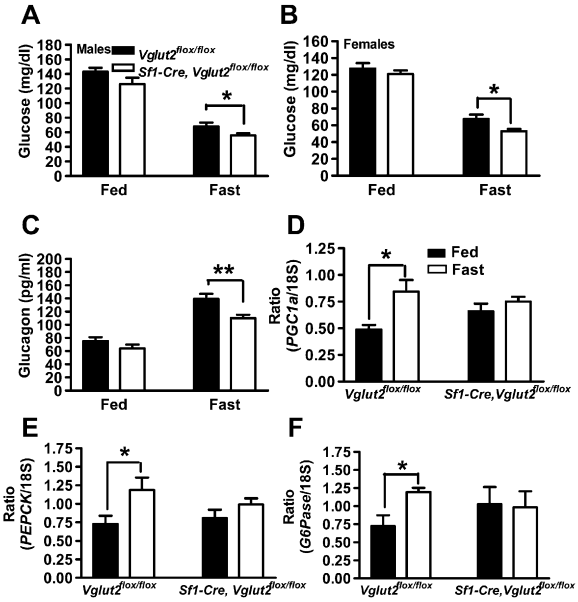


Figure 4. The Effects of Twenty-Four Hours of Fasting on Blood Glucose, Glucagon, and Liver Gene Expression

Male and female mice (n = 13–15 per group), age 8–10 weeks, were studied. *p < 0.05; **p < 0.01.

(A) Blood glucose in male mice.

(B) Blood glucose in female mice.

(C) Plasma glucagon in female mice.

(D–F) *PGC-1α*, *PEPCK*, and *G6Pase* mRNA levels in livers from female mice.

little or no role in the case of chow-fed mice and a small role in the case of fat-fed mice in regulating energy balance.

Impaired Glucose Homeostasis in the Fasted State

Sf1-Cre;Vglut2$^{flox/flox}$ mice had lower blood glucose levels, compared with *Vglut2*$^{flox/flox}$ controls, after fasting for 24 hr (55 ± 3 versus 68 ± 5 mg/dl in males, Figure 4A; 53 ± 2 versus 67 ± 5 mg/dl in females, Figure 4B). Insulin values fell with fasting in both groups and were similar in *Sf1-Cre;Vglut2*$^{flox/flox}$ mice versus *Vglut2*$^{flox/flox}$ mice in both the fed and fasted states (data not shown). In contrast, *Sf1-Cre;Vglut2*$^{flox/flox}$ mice had an impaired fasting-mediated increase in the pancreatic hormone glucagon (Figure 4C). Glucagon works on the liver, where it stimulates glycogenolysis (breakdown of glycogen to glucose) and gluconeogenesis (synthesis of glucose from pyruvate, lactate, glycerol, and amino acids) (Vidal-Puig and O'Rahilly, 2001). The effect on gluconeogenesis is mediated in part by augmenting expression of PGC-1α, a transcription coactivator that stimulates expression of genes encoding gluconeogenic enzymes such as glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) (Yoon et al., 2001). As expected, in livers of control *Vglut2*$^{flox/flox}$ mice, fasting increased expression of *Ppargc1α* (*PGC-1α*), *Pck1* (*PEPCK*), and *G6pc* (*G6Pase*) mRNAs (Figures 4D–4F). Of note, these fasting-induced increases in gene expression were absent in livers of *Sf1-Cre;Vglut2*$^{flox/flox}$ mice. These findings indicate that hypoglycemia during fasting in *Sf1-Cre;Vglut2*$^{flox/flox}$ mice is mediated at least in part by failure to increase blood glucagon levels and subsequent failure to induce hepatic expression of *PGC-1α* and its gluconeogenic targets, *G6Pase* and *PEPCK*.

Impaired Counterregulation of Insulin-Induced Hypoglycemia

Acute administration of insulin induces hypoglycemia. Counterregulatory measures, initiated in part by the brain, operate to limit the degree of hypoglycemia. As shown in Figure 5A, a single dose of insulin (1.5 U/kg) at 60 min caused a greater fall in blood glucose in *Sf1-Cre;Vglut2*$^{flox/flox}$ mice. This greater degree of hypoglycemia was associated with an impaired glucagon response (Figure 5B).

To confirm these findings, we performed hypoglycemic clamp studies, in which hypoglycemia is induced and sustained at similar levels in a controlled fashion (Figure 5C). *Sf1-Cre;Vglut2*$^{flox/flox}$ mice tended to require higher rates of glucose infusion during the clamp, which is consistent with decreased rates of endogenous glucose production (Figure 5D). Of note, hypoglycemia induced a large increase in plasma glucagon levels in control mice, and, importantly, this response was undetectable in *Sf1-Cre;Vglut2*$^{flox/flox}$ mice (Figure 5E). Similarly, hypoglycemia induced a large increase in blood levels of epinephrine in control mice, and this response was blunted in *Sf1-Cre;Vglut2*$^{flox/flox}$ mice (Figure 5F). In total, these results demonstrate that *Sf1-Cre;Vglut2*$^{flox/flox}$ mice have an impaired

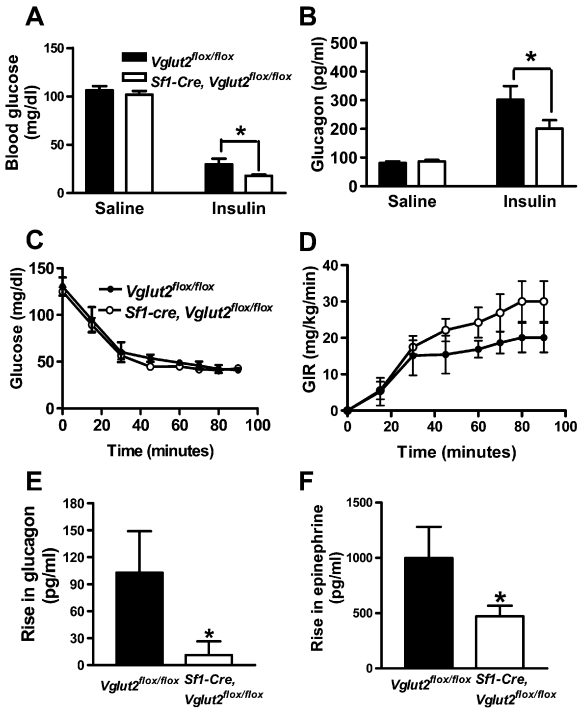


Figure 5. Effects of Insulin-Induced Hypoglycemia on Counterregulatory Response

For the single-injection study (A and B), male mice (n = 12–15), age 10–12 weeks, were fasted overnight and then given a single dose of insulin (1.5 U/kg). For the hyperinsulinemic-hypoglycemic clamp studies (C–F), male mice (n = 5–10), age 12–14 weeks, were used. Insulin infusion started at time 0 and continued during the entire 90 min period. Glucose was infused at a variable rate with the goal of maintaining glucose at approximately 45 mg/dl. *p < 0.05.

(A) Blood glucose at 60 min.

(B) Plasma glucagon at 60 min.

(C) Blood glucose levels during clamp.

(D) Glucose infusion rate (GIR) during the clamp.

(E) Rise in glucagon (increase at 60–90 min interval over level observed at time 0).

(F) Rise in epinephrine (increase at 60–90 min interval over level observed at time 0).

counterregulatory response to insulin-induced hypoglycemia.

Impaired Counterregulatory Response to Central 2-Deoxyglucose

2-deoxyglucose (2-DG) is a nonmetabolizable glucose analog that inhibits glucose metabolism in cells. Injection of 2-DG into the third ventricle of the brain is frequently used as a means of mimicking central glucopenia (Borg et al., 1995; Miki et al., 2001). This manipulation is useful because "glucopenia" is limited to the brain and also because it is not confounded by the presence of hyperinsulinemia. In control mice, central 2-DG treatment for 30 min increased blood glucose levels (Figure 6A), plasma glucagon levels (p = 0.06) (Figure 6B), and *PEPCK* mRNA levels in the liver (Figure 6C). There was also a trend for *G6Pase* mRNA to increase (Figure 6D). All of these responses to central 2-DG

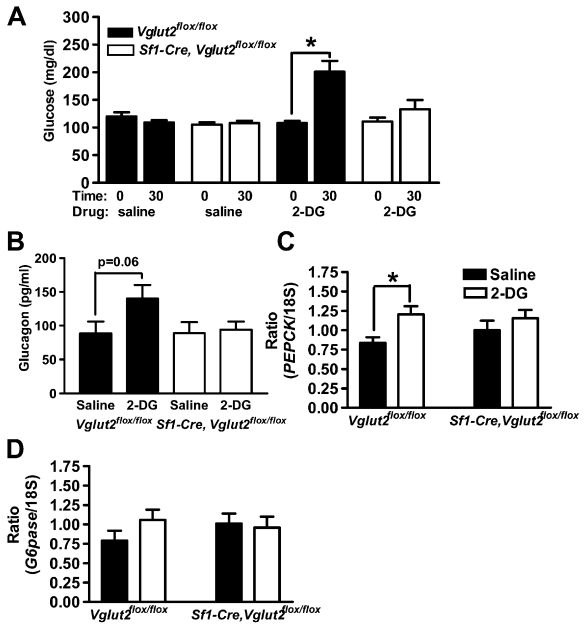


Figure 6. Effect of Centrally Administered 2-Deoxyglucose on Blood Glucose, Glucagon, and Liver Gene Expression

Female mice (n = 9–10 per group), age 10 weeks, were studied. 2-deoxyglucose (2-DG) or saline was administered i.c.v. *p < 0.05.

(A) Blood glucose was assessed at 0 and 30 min after saline or 2-DG treatment.

(B) Glucagon was assessed 30 min after saline or 2-DG treatment.

(C and D) *PEPCK* and *G6Pase* mRNA levels in the liver were assessed 30 min after saline or 2-DG treatment.

were absent in *Sf1-Cre;Vglut2*$^{flox/flox}$ mice. These results demonstrate that counterregulatory responses to central glucopenia are defective in *Sf1-Cre;Vglut2*$^{flox/flox}$ mice.

However, some counterregulatory responses may be normal in *Sf1-Cre;Vglut2*$^{flox/flox}$ mice. Peripheral 2-DG administration is also known to stimulate food intake (Ritter et al., 2006; Sindelar et al., 2004), which functions to raise blood glucose levels. The hyperphagic response to fasting or 2-DG was preserved in *Sf1-Cre;Vglut2*$^{flox/flox}$ mice (see Figure S3). These results suggest that glutamate release from SF1 neurons is not involved in the pathway by which central glucopenia stimulates food intake.

Induction of c-Fos in the Dorsal Motor Nucleus of the Vagus

Induction of c-Fos is a well-established indicator of neuronal activation (Herrera and Robertson, 1996). SF1 neurons release glutamate, which is an excitatory neurotransmitter, and this release is required for the glucagon response to hypoglycemia. Thus, there is an excitatory efferent pathway from SF1 neurons to pancreatic α cells. Neurons situated in this pathway activated (directly or indirectly) by glutamate released from SF1 neurons could be identified by comparing hypoglycemia-mediated c-Fos induction in *Vglut2*$^{flox/flox}$ mice versus *Sf1-Cre;Vglut2*$^{flox/flox}$ mice. Indeed, significantly more neurons express c-Fos in the dorsal motor nucleus of the vagus (DMV) of three consecutive

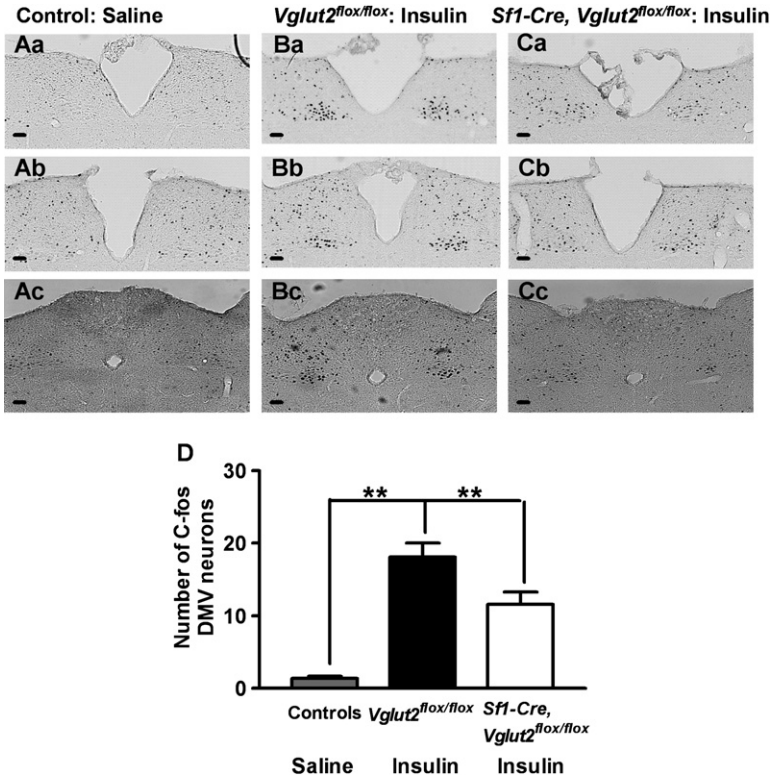


Figure 7. Induction of c-Fos in Dorsal Motor Nucleus of the Vagus

Sixty minutes after treatment with saline or insulin (1.5 U/kg), brains from *Vglut2*$^{flox/flox}$ and *Sf1-Cre;Vglut2*$^{flox/flox}$ mice were removed and immunostained for c-Fos. The saline-treated *Vglut2*$^{flox/flox}$ and *Sf1-Cre;Vglut2*$^{flox/flox}$ mice were pooled together as controls since c-Fos induction was not different between these mice. Three consecutive sections immediately rostral to the area postrema were analyzed. (n = 8 for control group; n = 4 for other groups.) Scale bar = 100 μM.

(Aa–Ac) c-Fos expression in saline-treated mice.

(Ba–Bc) c-Fos expression in insulin-treated *Vglut2*$^{flox/flox}$ mice.

(Ca–Cc) c-Fos expression in insulin-treated *Sf1-Cre;Vglut2*$^{flox/flox}$ mice.

(D) Summary of c-Fos-positive neurons in DMV nuclei.

sections immediately rostral to the area postrema in insulin-treated hypoglycemic *Vglut2*$^{flox/flox}$ mice (18 ± 2.0/DMV/section; Figures 7Ba–7Bc and 7D) compared to saline-treated mice (1.4 ± 0.1/DMV/section; Figures 7Aa–7Ac and 7D). In contrast, in insulin-treated *Sf1-Cre;Vglut2*$^{flox/flox}$ mice, the induction of c-Fos is significantly reduced (11 ± 1.7/DMV/section; Figures 7Ca–7Cc and 7D). Thus, it is likely that DMV neurons act as a link between SF1 neurons and pancreatic α cells.

DISCUSSION

Neuropeptides versus Fast-Acting Neurotransmitters

Important questions exist regarding roles of neuropeptides versus the fast-acting neurotransmitters glutamate and GABA in regulating hypothalamic responses (van den Pol, 2003). Neuropeptides and neurotransmitters may both function as independent transmitters, or, alternatively, neuropeptides may work by modulating the actions of glutamate and GABA. Key insight into the function of neuropeptides has been gained through the administration of agonists and antagonists, as well as the targeted disruption of genes encoding neuropeptides and their receptors. In contrast, with regards to assessing the roles of glutamate and GABA in hypothalamic function, these approaches have not been (or would not be) particularly informative. Thus, the role of fast-acting neurotransmitters in regulating hypothalamic function in vivo is largely unknown. Based upon the results of brain slice electrophysiology studies, it is likely that fast-acting neurotransmitters play important roles (Cowley et al., 1999, 2001; van den Pol, 2003). A major goal of the present study was to develop an approach for studying, in vivo, their roles in regulating behaviors and physiologic processes.

Toward these ends, we have used neuron-specific knockout of the vesicular glutamate transporter VGLUT2 to assess the role of glutamate. VGLUT2, but not VGLUT1 or VGLUT3, is expressed in the hypothalamus (Gras et al., 2002; Ziegler et al., 2002). The present study focused on the VMH, a site that is rich in glutamatergic neurons and thought to play important roles in controlling an array of behaviors and physiologic responses (Swanson, 1987). Specifically, we used Cre/lox technology to generate mice that lack VGLUT2 in SF1 neurons (*Sf1-Cre;Vglut2*$^{flox/flox}$ mice). SF1 neurons constitute the majority of VMH neurons (Stallings et al., 2002). Electrophysiologic studies confirmed that glutamatergic transmission by SF1 neurons was completely disrupted by this approach (Figure 2C). Thus, *Sf1-Cre;Vglut2*$^{flox/flox}$ mice should be extremely useful for determining the function of glutamate release by VMH neurons. This general approach (neuron-specific deletion of vesicular transporters) and the generated mouse lines (for example, *Vglut2*$^{flox/flox}$ mice) should have broad applicability to studies where the goal is to link neurotransmission by selected neurons with specific behaviors and physiologic responses.

Counterregulatory Response to Hypoglycemia and the Role of the Brain

The brain requires a continuous supply of glucose for proper function and survival. To prevent hypoglycemia,

low blood glucose elicits the following adaptive responses (Cryer, 2005): (1) insulin secretion by pancreatic β cells decreases; (2) glucagon secretion by pancreatic α cells increases; (3) if needed, epinephrine secretion by the adrenal medulla gland increases; and (4) food intake is stimulated. Reduced insulin secretion is thought to be a direct response of β cells to low blood glucose. The other responses, however, are driven primarily by the brain. These critically important countermeasures are initiated by fasting, and iatrogenically, when hypoglycemia develops following insulin treatment. Indeed, insulin-induced hypoglycemia complicates and severely limits the widespread application of intensive insulin therapy in type 1 diabetes, which has been shown to markedly reduce microvascular complications of chronic hyperglycemia (Cryer, 2005). This issue is further exacerbated by the fact that counterregulatory responses to hypoglycemia are defective in individuals with diabetes (Cryer, 2005). For this reason, understanding the mechanisms responsible for counterregulatory responses and the reasons why they are inoperative in diabetics is an extremely important area for investigation.

The CNS detects hypoglycemia and then initiates counterregulatory responses. The brain contains "glucose-excited" neurons (whose firing rate increases as glucose levels rise) and "glucose-inhibited" neurons (whose firing rate increases as glucose levels fall) (Anand et al., 1962; Burdakov et al., 2005b; Oomura et al., 1974; Routh, 2002). Glucose depolarizes glucose-excited neurons by closing ATP-sensitive K^+ channels (Ashford et al., 1990; Miki et al., 2001; Routh, 2002). The mechanism by which glucose hyperpolarizes glucose-inhibited neurons, on the other hand, is less well understood, but it may involve altered activity of chloride channels (Routh, 2002) or Na/K pumps (Silver and Erecinska, 1998), or could also involve tandem-pore K^+ (K_{2p}) channels, as was recently shown for glucose-inhibited orexin neurons (Burdakov et al., 2006). Neurons capable of sensing glucose are abundant in various parts of the hypothalamus (including the VMH, the lateral hypothalamus, and the arcuate nucleus) and in the hindbrain (Burdakov et al., 2005a; Ibrahim et al., 2003; Marty et al., 2005; Muroya et al., 1999; Ritter et al., 2006; Routh, 2002). In principle, the method employed by the brain to detect hypoglycemia could involve decreased activity of glucose-excited neurons, increased activity of glucose-inhibited neurons, input from peripheral blood glucose sensors (Burcelin et al., 2000), or possibly some combination of these three processes.

The Role of the VMH

The neurocircuitry underlying detection of hypoglycemia and subsequent counterregulatory responses is not well known. The VMH is believed to play a role for the following reasons: (1) the VMH contains neurons capable of sensing glucose (Ashford et al., 1990; Routh, 2002); (2) local glucopenia in the VMH induced by infusion of 2-DG produces counterregulatory responses (Borg et al., 1995); (3) lesions of the VMH impair counterregulatory responses (Borg et al., 1994); and (4) infusion of glucose directly into the VMH during systemic hypoglycemia attenuates counterregulatory responses (Borg et al., 1997). While these findings implicate the VMH, they are limited by uncertainty regarding the range of diffusion of infused molecules (glucose and 2-DG) and the extent of ablative lesions. Of note, glucose-excited (POMC and MCH) neurons and glucose-inhibited (NPY/AGRP and orexin) neurons are found nearby in the arcuate nucleus and the lateral hypothalamus (Burdakov et al., 2005a; Ibrahim et al., 2003; Muroya et al., 1999).

In the present study, we have shown that *Sf1-Cre; Vglut2*$^{flox/flox}$ mice have clear defects in counterregulatory responses. This manifests itself as hypoglycemia during fasting and insulin treatment and as a defective hyperglycemic response to neuroglycopenia (induced by central infusion of 2-DG). The greatest counterregulatory defect was seen in hypoglycemia-induced secretion of glucagon. A modest impairment was seen in hypoglycemia-induced secretion of epinephrine. Of note, no impairment was seen in the hyperphagic response to neuroglycopenia. This latter finding is in agreement with studies implicating hindbrain catecholaminergic cell groups with projection to the PVH (Ritter et al., 2006) and neuropeptide Y (Sindelar et al., 2004), which is not found in the VMH, in mediating neuroglycopenia-induced hyperphagia. In total, the present study definitively establishes a role for SF1 neurons and glutamate release by these neurons in the homeostatic neurocircuitry that prevents hypoglycemia.

Afferents and Efferents of SF1 Neurons

The "first-order neurons" responsible for detecting hypoglycemia have not been identified. They could be the SF1 neurons themselves. A subset of VMH neurons are known to sense glucose (Song and Routh, 2005), some of which could be SF1 neurons. Alternatively, it is possible that the primary "glucose-sensing" neurons are upstream of SF1 neurons. One possibility is that the hindbrain catecholaminergic neurons sense glucose. These neurons are responsive to hypoglycemia and send projections to the VMH (Ritter et al., 2006). Consistent with this, the norepinephrine level is increased in the VMH area in response to hypoglycemia (Beverly et al., 2001), and VMH neurons express adrenergic receptors (Boundy and Cincotta, 2000). Another possibility is that the orexin neurons in the lateral hypothalamus sense glucose; these neurons have been shown to be excited by low glucose (Burdakov et al., 2005a, 2006). Orexin receptors 1 (OX_1R) and 2 (OX_2R) are both expressed at high levels in the VMH (Marcus et al., 2001). Other neurons, including glucose-excited POMC neurons and MCH neurons or glucose-inhibited NPY/AGRP neurons, could also be upstream of SF1 neurons (Burdakov et al., 2005a; Ibrahim et al., 2003; Muroya et al., 1999; Ritter et al., 2006). Likewise, it is possible that peripheral glucose sensors (Burcelin et al., 2000) could provide input to the VMH. Identifying the primary site responsible for detecting hypoglycemia and determining how this site connects with SF1 neurons is an important area of investigation.

Similarly, the efferent pathways connecting SF1 neurons with glucagon secretion are also of interest. The autonomic

nervous system controls glucagon secretion, and, importantly, blockade of parasympathetic and sympathetic input to pancreatic α cells prevents hypoglycemia-induced and neuroglycopenia-induced increases in glucagon (Rossi et al., 2005; Taborsky et al., 1998). Given this, parasympathetic preganglionic neurons in the DMV and sympathetic preganglionic neurons in the spinal cord could be downstream of SF1 neurons. Consistent with an important role for parasympathetic preganglionic neurons, we have found that hypoglycemia-mediated induction of c-Fos in the DMV at the level of the area postrema is significantly attenuated in *Sf1-Cre;Vglut2*$^{flox/flox}$ mice (Figure 7). This finding is likely to be relevant since DMV neurons at this level send direct projections to the pancreas (Rinaman and Miselis, 1987). How SF1 neurons connect with DMV neurons, however, remains to be determined. The connection may be indirect since direct projections from the VMH to the DMV have yet to be described (Canteras et al., 1994).

Body Weight and Release of Glutamate from SF1 Neurons

Mice homozygous for a disrupted *Sf1* allele and mice lacking leptin receptors on SF1 neurons (*Sf1-Cre;Lepr*$^{flox/flox}$ mice) are modestly obese when fed a chow diet and develop marked obesity when fed a high-fat diet (Dhillon et al., 2006; Majdic et al., 2002). With this in mind, it is surprising that *Sf1-Cre;Vglut2*$^{flox/flox}$ mice do not become obese when fed a chow diet and have very limited obesity when fed a high-fat diet. These findings raise the possibility that some other effector released from SF1 neurons, for example BDNF (Xu et al., 2003) or GABA (as shown in Figure 2, some SF1 neurons are GABAergic), may play a role in regulating body weight. We suspect that BDNF release from SF1 neurons is not involved since mice lacking BDNF in SF1 neurons (*Sf1-Cre;Bdnf*$^{flox/flox}$ mice) failed to develop obesity (data not shown). We are presently testing the role of GABA by assessing energy homeostasis in *Sf1-Cre;Vgat*$^{flox/flox}$ mice.

Summary

This study demonstrates that neuron-specific manipulation of vesicle neurotransmitter transporters, such as VGLUT2, is a useful means of linking fast-acting neurotransmitter release with specific functions. In addition, we have established that SF1 neurons in the VMH and glutamate release by these neurons are important components of the neurocircuitry that functions to prevent hypoglycemia. Using SF1 neurons as a starting point, it will be important to identify the afferent and efferent components of this circuitry. Knowledge of these pathways should provide a framework for studies aimed at understanding the clinically significant problem of defective counterregulatory responses to hypoglycemia in individuals with diabetes.

EXPERIMENTAL PROCEDURES

Animal Care

All animal care and experimental procedures were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. Mice were housed at 22°C–24°C with a 14 hr light/10 hr dark cycle with standard mouse chow (Teklad F6 Rodent Diet 8664; 4.05 kcal/g, 3.3 kcal/g metabolizable energy, 12.5% kcal from fat; Harlan Teklad) and water provided ad libitum.

Generation of *Vglut2*$^{flox/flox}$ Mice and *Sf1-Cre;Vglut2*$^{flox/flox}$ Mice

The lox-modified *Vglut2* (*Slc17a6*) targeting construct was constructed using a mouse 129 BAC genomic clone and recombineering technology (Lee et al., 2001). The two *loxP* sites were inserted in the first intron and the second intron, respectively.

Sf1-Cre;Vglut2$^{+/flox}$ mice were generated by mating *Sf1-Cre* transgenic mice (FVB/N background) (Dhillon et al., 2006) with *Vglut2*$^{+/flox}$ mice (129, C57BL/6 background), and study subjects were generated by mating *Sf1-Cre;Vglut2*$^{flox/flox}$ mice with *Vglut2*$^{flox/flox}$ mice. See Supplemental Experimental Procedures for details.

High-Fat, High-Sucrose Diet Study

Groups of 8- to 10-week-old male controls (*Vglut2*$^{flox/flox}$ mice) and *Sf1-Cre;Vglut2*$^{flox/flox}$ mice were switched to a high-fat, high-sucrose diet (HFD) and maintained on HFD for 12 weeks. See Supplemental Experimental Procedures for study details.

Electrophysiologic Recordings on Autaptic Cultured Neurons

Micropunched VMH tissues were digested using a papain dissociation system (Worthington Biochemical Corporation). Digested neurons were seeded on poly-L-lysine-coated glass coverslips in Neurobasal A medium (Invitrogen). The neurons were planted at low density to encourage the formation of autosynapses.

Synaptic currents were recorded in whole-cell voltage-clamp mode, and release of synaptic vesicles was evoked by a depolarizing pulse to +10 mV from the holding potential of −60 mV, applied every 20 s. The solution used in the recording pipette permits identification of both glutamatergic EPSCs and GABAergic IPSCs as inward current. EPSCs and IPSCs were identified by their deferent decay time constants and by using specific glutamate receptor (QBNX and D-APV) and GABA receptor (bicuculline) antagonists. See Supplemental Experimental Procedures for details.

In Situ Hybridization Studies

In situ hybridizations were performed as previously described (Kishi et al., 2003). See Supplemental Experimental Procedures for details.

Glucose Homeostasis Studies

Fed blood glucose levels were measured between 9 a.m. and 10 a.m. Fasted blood glucose, liver gluconeogenic gene expression, and glucagon levels were measured after 24 hr fasting.

For the insulin-induced hypoglycemia study, overnight-fasted males were administrated i.p. with saline or insulin at 1.5 U/kg. At 1 hr after administration, blood was collected for determination of various parameters.

For i.c.v. injections, a stainless-steel cannula (30 gauge) was implanted in the right lateral ventricle. On the test day, 2-deoxyglucose (1 mg/3 μl vol/mouse) or saline was injected i.c.v. into fed mice. At 30 min after injection, liver samples were collected, and glucose, insulin, and glucagon were determined. See Supplemental Experimental Procedures for details.

Quantitative PCR Assay

Liver RNA was reverse transcribed with RETROscript (Ambion, Inc.) and amplified using Assay-on-Demand TaqMan probes and primers (Applied Biosystems). Quantitative PCR was performed on an Mx4000 instrument (Stratagene). See Supplemental Experimental Procedures for details.

Hyperinsulinemic-Hypoglycemic Clamp Studies

The hyperinsulinemia-hypoglycemia protocol was a modification of a procedure previously described for rats (McCrimmon et al., 2006). Briefly, at t = 0, a 90 min 10 mU/kg/min infusion of human regular

insulin (Eli Lilly) was begun. Glucose was allowed to fall to ~45 mg/dl and was then maintained at this level for 60–90 min. Samples for measurement of the hormones epinephrine, norepinephrine (HPLC with electrochemical detection), and glucagon (RIA, Linco Research Inc.) were taken at 0, 60, and 90 min. See Supplemental Experimental Procedures for details.

Hypoglycemia-Mediated c-Fos Induction

Vglut2$^{flox/flox}$ and *Sf1-Cre;Vglut2*$^{flox/flox}$ mice were acclimated for 7 days before i.p. injection of either saline (four mice from each genotype) or insulin (1.5U/kg, four mice from each genotype). Blood glucose was then monitored, and the mice were transcardially perfused at 60 min. The brains were cut in five series with 25 μM thickness and immunostained for c-Fos with α-c-Fos (catalog # PC38 [Calbiochem] raised in rabbit at 1:25,000).

In the DMV, the greatest induction of c-Fos was observed in three consecutive sections immediately rostral to the appearance of the area postrema in *Vglut2*$^{flox/flox}$ mice. Therefore, c-Fos-positive neurons were counted in the DMV of these sections. The saline-treated *Vglut2*$^{flox/flox}$ mice and *Sf1-Cre;Vglut2*$^{flox/flox}$ mice were pooled together as a saline control, as there was no difference in c-Fos induction in these mice. Only neurons showing clear, round c-Fos-stained nuclei were counted as positive for c-Fos.

Statistical Methods

Data sets were analyzed for statistical significance using Prism (GraphPad) for a two-tailed unpaired Student's t test.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and three figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/5/5/383/DC1/.

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